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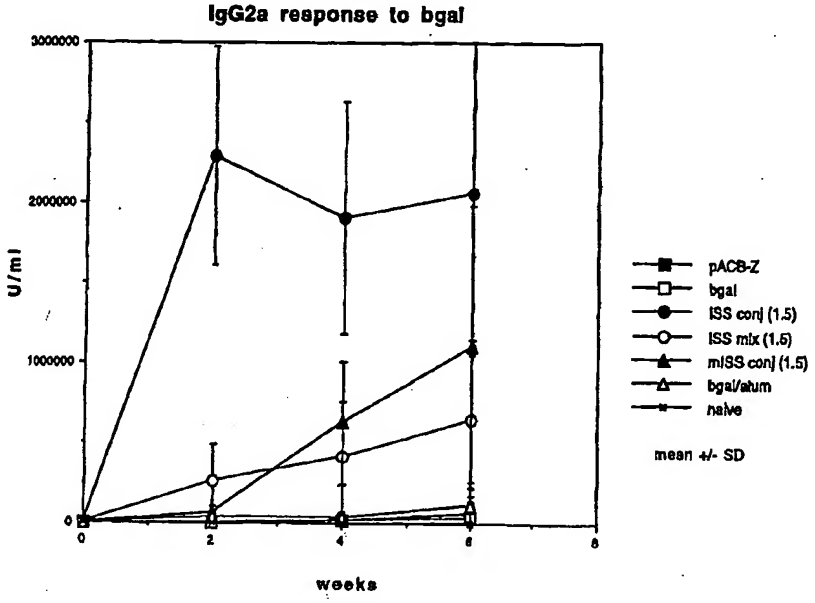

(57) Abstract

Immunostimulatory polynucleotide–immunomodulatory molecule conjugate compositions are disclosed. These compositions include a polynucleotide that is linked to an immunomodulatory molecule, which molecule comprises an antigen and may further comprise immunomodulators such as cytokines and adjuvants. The polynucleotide portion of the conjugate includes at least one immunostimulatory oligonucleotide nucleotide sequence (ISS). Methods of modulating an immune response upon administration of the polynucleotide–immunomodulatory conjugate preparation to a vertebrate host are also disclosed.

IMMUNOSTIMULATORY POLYNUCLEOTIDE/IMMUNOMODULATORY MOLECULE CONJUGATES

RELATED U.S. PATENT APPLICATIONS

This is a continuation-in-part and utility conversion of U.S. Provisional Patent Application Serial No. 60/028,118, filed October 11, 1996.

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FIELD OF THE INVENTION

The invention relates to compositions comprising an immunomodulatory molecule (IMM) including an antigen, conjugated to a polynucleotide that contains or consists of at least one immunostimulatory oligonucleotide (ISS-PN). It also relates to methods for modulating the immune response of a vertebrate host to an antigen.

SUMMARY OF THE INVENTION

The present invention provides compositions comprising an ISS-PN which is conjugated to an IMM (which includes an antigen) to form ISS-PN/IMM conjugates. The ISS-PN/IMM conjugates of the invention are biological response modifiers in the sense that they modify the humoral and cellular immune response of a host to an antigen.

Specifically, the ISS-PN and IMM components of the ISS-PN/IMM conjugates synergistically boost the magnitude of the host immune response against an antigen to a level greater than the host immune response to either the IMM, antigen or ISS-PN alone. The ISS-PN/IMM conjugates also shift the host cellular immune response away from the helper T lymphocyte type 2 (Th2) phenotype toward a helper T lymphocyte type 1 (Th1) phenotype. These responses to ISS-PN/IMM conjugates are particularly acute during the important early phase of the host immune response to an antigen.

To these ends, ISS-PN/IMM conjugates are delivered by any route through which antigen-sensitized host tissues will be contacted with the ISS-PN/IMM conjugate. ISS-PN/IMM conjugates administered in this fashion boost both humoral (antibody) and cellular (Th1 type) immune responses of the host. Thus, use of the method to boost the immune responsiveness of a host to subsequent challenge by a sensitizing antigen without immunization avoids the risk of Th2-mediated, immunization-induced anaphylaxis by suppressing IgE production in response to the antigen challenge. An especially advantageous use for this aspect of the invention is treatment of localized allergic responses in target tissues where the allergens enter the body, such as the skin and mucosa.

Suppression of the Th2 phenotype according to the invention is also a useful in reducing antigen-stimulated IL-4 and IL-5 production. Thus, the invention encompasses delivery of ISS-PN/IMM conjugates to a host to suppress the Th2

In one aspect of the invention, the ISS-PN consists of an ISS-ODN. Alternatively, the ISS-PN comprises an ISS-ODN.

Conjugates of the invention also include PN/IMM wherein the PN serves as a carrier to introduce the IMM antigen into MHC Class I processing pathways not normally stimulated by soluble antigen, but lacks ISS activity and therefore does not stimulate a Th1 phenotype immune response. Examples of such PN/IMM are those wherein the CpG motif is mutated, for example, to a GpG motif.

In one aspect of the invention, the IMM conjugate partner to the ISS-PN consists of an antigen. Such antigens are selected from the group of antigens consisting of proteins, peptides, glycoproteins, polysaccharides and gangliosides.

In another aspect of the invention, the IMM conjugate partner comprises an antigen and further comprises an immunostimulatory molecule selected from the group of such molecules consisting of adjuvants, hormones, growth factors, cytokines, chemokines, targeting protein ligands, and trans-activating factors.

In another aspect of the invention, the ISS-PN/IMM conjugate is modified for targeted delivery by, for example, attachment to a monoclonal antibody, receptor ligand and/or liposome.

Pharmaceutically acceptable compositions of ISS-PN/IMM conjugates are provided for use in practicing the methods of the invention. Where appropriate to the contemplated course of therapy, the ISS-PN/IMM conjugates may be administered with anti-inflammatory or immunotherapeutic agents. Thus, a particularly useful composition for use in practicing the method of the invention is one in which an anti-inflammatory agent (e.g., a glucocorticoid) is mixed with, or further conjugated to, an ISS-PN/IMM conjugate.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is a graph of data demonstrating the vigorous Th1-type immune response (as measured by production of IgG2a against an IMM antigen) stimulated by ISS-PN/IMM (1:5 ratio) in comparison to the levels of Th2-like responses stimulated by an ISS containing, antigen encoding plasmid (pACB-Z); the antigen alone (β-gal); the antigen mixed with an ISS (1:5 ratio); the antigen conjugated to a non-stimulatory PN (mISS conj; 1:5 ratio); the antigen in adjuvant (alum) and, for reference, the IgG2a levels in naive (unexposed) mice. The horizontal axis represents the levels (units/ml) of antibody; the vertical axis represents the number of weeks following primary antigen exposure.

FIGURE 2 is a graph of data demonstrating the levels of Th2-type immune responses (as measured by production of IgG1 against an IMM antigen) stimulated by an ISS containing, antigen encoding plasmid (pACB-Z); the antigen alone (β-gal); the antigen mixed with an ISS (1:5 ratio); the antigen conjugated to a non-stimulatory PN (mISS conj; 1:5 ratio); the antigen in adjuvant (alum) and, for reference, the IgG1 levels in naive (unexposed) mice, all as compared to the vigorous Th1-type immune response produced in mice immunized with ISS-PN/IMM (1:5 ratio). The horizontal axis represents the levels (units/ml) of antibody; the vertical axis represents the number of weeks following primary antigen exposure.

FIGURE 3 is a graph of data demonstrating the vigorous Th1-type immune response (as measured by production of IgG2a against an IMM antigen) stimulated by ISS-PN/IMM in comparison to the levels of Th2-like responses stimulated by the antigen alone (AgE) and antigen conjugated to a non-stimulatory PN (mISS conj). Antigen to PN ratios are all 1:5. The horizontal axis represents the levels (units/ml) of antibody; the vertical axis shows the levels at 4 weeks following primary antigen exposure (shaded bars) and at 2 weeks following secondary antigen challenge (solid bars).

Z); the antigen alone (β-gal); the antigen mixed with an ISS; the antigen conjugated to a non-stimulatory PN (mISS conj); the antigen in adjuvant (alum) and, for reference, the CTL levels in naive (unexposed) mice. Antigen to PN ratios are all 1:5. The horizontal axis represents the levels of antigen-specific cell lysis obtained (as a percentage of control; no antigen); the vertical axis shows the levels of CTL detected at different effector (antigen) to target ratios, from 0:1 to 10:1. The legend identifies how each cell population was treated.

Active Th1 (IFNγ) cells enhance cellular immunity and are therefore of particular value in responding to intracellular infections, while active Th2 cells enhance antibody production and are therefore of value in responding to extracellular infections (at the risk of anaphylactic events associated with IL-4 stimulated induction of IgE antibody production). Thus, the ability to shift host immune responses from the Th1 to the Th2 repertoire and vice versa has substantial clinical significance for controlling host immunity against antigen challenge (e.g., in infectious and allergic conditions).

To that end, the methods of the invention shift the host immune response to a sensitizing antigen toward a Th1 phenotype (Example I). Consequently, Th2 associated cytokine production and antigen stimulated production of IgE (Examples II and III) are suppressed, thereby reducing the host's risk of prolonged allergic inflammation and minimizing the risk of antigen-induced anaphylaxis. CTL production is also stimulated to a greater degree in animals treated according to the invention. Because CTL production is tied to antigen processing in Class I MHC pathways, increased CTL production can be produced from non-immunostimulatory PN/IMM as well as ISS-PN/IMM (Example IV).

Although the invention is not limited to any particular mechanism of action, it is conceivable that PN facilitate uptake of exogenous antigen by antigen presenting cells for presentation through host MHC Class I processing pathways not normally stimulated by soluble antigen. Thus, ISS-PN/IMM carry antigen into MHC Class I processing pathways (which may also be achieved by PN/IMM without ISS activity) then stimulate a cytokine cascade in a Th1 phenotype (as a result of ISS activity). Whatever the mechanism of action, use of ISS-PN/IMM to boost the host's immune responsiveness to a sensitizing antigen and shift the immune response toward a Th1 phenotype avoids the risk of immunization-induced anaphylaxis, suppresses IgE production in response to a sensitizing antigen and eliminates the need to identify the sensitizing antigen for use in immunization.

plasmid. The term "polynucleotide" therefore includes oligonucleotides, modified oligonucleotides and oligonucleosides, alone or as part of a larger construct. The polynucleotide may be single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA).

The polynucleotide portion can be linearly or circularly configured, or the oligonucleotide portion can contain both linear and circular segments. Modifications of oligonucleotides include, but are not limited to, modifications of the 3'OH or 5'OH group, modifications of the nucleotide base, modifications of the sugar component, and modifications of the phosphate group.

The oligonucleotide base of ISS-PN/IMM conjugates may comprise ribonucleotides (containing ribose as the only or principal sugar component), deoxyribonucleotides deoxyribose as the principal sugar component), or in accordance with established state-of-the-art modified sugars or sugar analogs may be incorporated in the oligonucleotide of the present invention. Thus, in addition to ribose and deoxyribose, the sugar moiety may be pentose, deoxypentose, hexose, deoxyhexos, glucose, arabinose, xylose, lyxose, and a sugar "analog" cyclopentyl group. The sugar may be in pyranosyl or in a furanosyl form. In the modified oligonucleotides of the present invention the sugar moiety is preferably the furanoside of ribose, deoxyribose, arabinose or 2'-0-methylribose, and the sugar may be attached to the respective heterocyclic bases either in I or J anomeric configuration. The preparation of these sugars or sugar analogs and the respective "nucleosides" wherein such sugars or analogs are attached to a heterocyclic base (nucleic acid base) per se is known, and need not be described here, except to the extent such preparation may pertain to any specific example.

The phosphorous derivative (or modified phosphate group) which may be attached to the sugar or sugar analog moiety in the modified oligonucleotides of the present invention may be a monophosphate, diphosphate, triphosphate, alkylphosphate,

Some oligonucleotide ISS (ISS-ODN) are known. In such ISS-ODN, the CpG motif is flanked by at least two purine nucleotides (e.g., GA or AA) and at least two pyrimidine nucleotides (5'-Purine-Purine-[C]-[G]-Pyrimidine-Pyrimidine-3'). CpG motif-containing ISS-ODN are believed to stimulate B lymphocyte proliferation (see, e.g., Krieg, *et al.*, *Nature*, 374:546-549, 1995).

The core hexamer structure of the foregoing ISS-PN may be flanked upstream and/or downstream by any number or composition of nucleotides or nucleosides. However, ISS-PN are at least 6 bases in length, and preferably are between 6 and 200 bases in length, to enhance uptake of the ISS-PN/IMM into target tissues. Those of ordinary skill in the art will be familiar with, or can readily identify, reported nucleotide sequences of known ISS-ODN for reference in preparing ISS-PN. For ease of reference in this regard, the following sources are especially helpful:

Yamamoto, *et al.*, *Microbiol.Immunol.*, 36:983 (1992)
Ballas, *et al.*, *J.Immunol.*, 157:1840 (1996)
Klinman, *et al.*, *J.Immunol.*, 158:3635 (1997)
Sato, *et al.*, *Science*, 273:352 (1996)

Each of these articles are incorporated herein by reference for the purpose of illustrating the level of knowledge in the art concerning the nucleotide composition of known ISS-ODN .

In particular, ISS-PN and PN useful in the invention include those which have the following hexameric nucleotide sequences:

1. For ISS-PN, hexamers having "CpG" motifs or, for PN, hexamers having XpY motifs, where X cannot be C if Y is G and vice-versa; and,

properties, phosphorothioates and phosphorodithioates are more resistant to degradation *in vivo* than their unmodified oligonucleotide counterparts, making the ISS-PN/IMM of the invention more available to the host.

2. IMM conjugate partners.

The oligonucleotide base of the ISS-PN/IMM conjugate is conjugated to an IMM which includes an antigen and may further include an immunomodulatory agent. An "antigen" is a substance that is recognized and bound specifically by an antibody or by a T cell antigen receptor. Antigens can include peptides, proteins, glycoproteins and polysaccharides, including portions thereof and combinations thereof. The antigens can be those found in nature or can be synthetic.

The term "immunomodulatory" as used herein includes immunostimulatory as well as immunosuppressive effects. Immunostimulatory effects include, but are not limited to, those that directly or indirectly enhance cellular or humoral immune responses. Examples of immunostimulatory effects include, but are not limited to, increased antigen-specific antibody production; activation or proliferation of a lymphocyte population such as NK cells, CD4+ T lymphocytes, CD8+ T lymphocytes, macrophages and the like; as well as increased synthesis of Th1 associated immunostimulatory cytokines including, but not limited to, IL-6, IL-12, IL-18, IFN-α,β and γ, TNF-α and the like. Immunosuppressive effects include those that directly or indirectly decrease cellular or humoral immune responses.

Examples of immunosuppressive effects include, but are not limited to, a reduction in antigen- specific antibody production such as reduced IgE production; activation of lymphocyte or other cell populations that have immunosuppressive activities such as those that result in immune tolerance; and increased synthesis of cytokines that have suppressive effects toward certain cellular functions. One example of this is IFN-γ, which can block IL-4 induced class switch to IgE and IgG1, thereby reducing the

More specifically, suitable antigens for use as ISS-PN/IMM conjugate partners include any molecule capable of being conjugated to an oligonucleotide and eliciting a B cell or T cell antigen-specific response. Preferably, antigens elicit an antibody response specific for the antigen. A wide variety of molecules are antigens. These include, but are not limited to, sugars, lipids, autacoids and hormones, as well as macromolecules such as complex carbohydrates, and phospholipids. Small molecules may need to be haptenized in order to be rendered antigenic.

Preferably the antigens are peptides, polysaccharides (such as the capsular polysaccharides used in *Haemophilus influenza* vaccines), gangliosides and glycoproteins. The antigen may be an intact antigen or T cell epitope(s) of an antigen. These can be obtained through several methods known in the art, including isolation and synthesis using chemical and enzymatic methods. In certain cases, such as for many sterols fatty acids and phospholipids, the antigenic portions are commercially available.

Many antigenic peptides and proteins are known in, and available to the art; others can be identified using conventional techniques. Examples of known antigens include, but are not limited to :

a. Allergens such as reactive major dust mite allergens *Der pI* and *Der pII* (*see,* Chua, *et al., J.Exp.Med.*, 167:175-182, 1988; and, Chua, *et al., Int.Arch.Allergy Appl. Immunol.*, 91:124-129, 1990), T cell epitope peptides of the *Der pII* allergen (*see,* Joost van Neerven, *et al., J.Immunol.*, 151:2326-2335, 1993), the highly abundant Antigen E (*Amb al*) ragweed pollen allergen (*see,* Rafnar, *et al., J.Biol.Chem.*, 266:1229-1236, 1991), phospholipase A_2 (bee venom) allergen and T cell epitopes therein (*see,* Dhillon, *et al., J.Allergy Clin.Immunol.*, __:42-__, 1992), white birch pollen (*Betvl*) (*see,* Breiteneder, *et al., EMBO,* 8:1935-1938, 1989), the *Fel dI* major domestic cat allergen (*see,* Rogers, *et al., Mol.Immunol.*, 30:559-568, 1993), tree pollen (*see,* Elsayed et al., *Scand. J. Clin. Lab. Invest. Suppl.*, 204:17-31,

Vaccine, vol. 10, 1000 (1992); Pockley, A.G. & Montgomery, P.C., "In vivo Adjuvant Effect of Interleukins 5 and 6 on Rat Tear IgA Antibody Responses" *Immunology*, vol. 73, 19-23 (1991); Adam, A. & Lederer, E. "Muramyl peptides as Immunomodulators" ISI ATLAS OF SCIENCE 205 (1988); Clements, J.D., et al. "Adjuvant Activity of Escherichia coli Heat-labile Enterotoxin and Effect on the Induction of Oral Tolerance in Mice to Unrelated Protein Antigens" *Vaccine*, vol. 6, 269 (1988); Ben Ahmeida, E.T.S., *et al.* "Immunopotentiation of Local and Systemic Humoral Immune Responses by ISCOMs, Liposomes and FCA: Role in Protection Against Influenza A in Mice" *Vaccine*, vol. 11, 1302 (1993); and Gupta, R.K. *et al.* "Adjuvants -- A Balance Between Toxicity and Adjuvanticity" *Vaccine*, vol. 11, 290-308 (1993).

Those of ordinary skill in the art will appreciate that non-antigen components of IMM described above can also be administered in unconjugated form with an ISS-PN/IMM (antigen only) conjugate. Thus, the co-administration of such components is encompassed by the invention.

C. *Synthesis of Polynucleotide Conjugates*

1. Polynucleotide portion

ISS-PN can be synthesized using techniques and nucleic acid synthesis equipment which are well-known in the art. For reference in this regard, see, e.g., Ausubel, *et al.*, *Current Protocols in Molecular Biology,* Chs. 2 and 4 (Wiley Interscience, 1989); Maniatis, *et al., Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., New York, 1982); U.S. Patent No. 4,458,066 and U.S. Patent No. 4,650,675. When assembled enzymatically, the individual units can be ligated with a ligase such as T4 DNA or RNA ligase as described in, for example, U.S. Patent No. 5,124,246. Oligonucleotide degradation could be accomplished through the exposure of an oligonucleotide to a nuclease, as exemplified in U.S. Patent No. 4,650,675. These references are incorporated herein by reference for the sole purpose of demonstrating knowledge in the art concerning production of synthetic polynucleotides. Because the

The preparation of base-modified nucleosides, and the synthesis of modified oligonucleotides using said base-modified nucleosides as precursors, has been described, for example, in U.S. Patents 4,910,300, 4,948,882, and 5,093,232. These base-modified nucleosides have been designed so that they can be incorporated by chemical synthesis into either terminal or internal positions of an oligonucleotide. Such base-modified nucleosides, present at either terminal or internal positions of an oligonucleotide, can serve as sites for attachment of a peptide or other antigen. Nucleosides modified in their sugar moiety have also bee described (e.g., U.S. Patents 4,849,513, 5,015,733, 5,118,800, 5,118,802) and can be used similarly.

The techniques for making phosphate group modifications to oligonucleotides are known in the art and do not require detailed explanation. For review of one such useful technique, the an intermediate phosphate triester for the target oligonucleotide product is prepared and oxidized to the naturally occurring phosphate triester with aqueous iodine or with other agents, such as anhydrous amines. The resulting oligonucleotide phosphoramidates can be treated with sulfur to yield phophorothioates. The same general technique (excepting the sulfur treatment step) can be applied to yield methylphosphoamidites from methylphosphonates. For more details concerning phosphate group modification techniques, those of ordinary skill in the art may wish to consult U.S. Patent Nos. 4,425,732; 4,458,066; 5,218,103 and 5,453,496, as well as *Tetrahedron Lett.* at 21:4149 (1995), 7:5575 (1986), 25:1437 (1984) and *Journal Am. ChemSoc.*, 93:6657 (1987), the disclosures of which are incorporated herein for the sole purpose of illustrating the standard level of knowledge in the art concerning preparation of these compounds.

2. Linking the PN component to the IMM component

The ISS-PN component can be linked to the IMM portion of the conjugate in a variety of ways. The link can be made at the 3' or 5' end of the ISS-PN, or to a suitably modified base at an internal position in the PN. If the peptide contains a suitable

Nucleic Acids Res. (1986) 14:6227-6245; Connolly, Nucleic Acids Res. (1985) 13:4485-4502; Coull et al., Tetrahedron Lett. (1986) 27:3991-3994; Kremsky et al., Nucleic Acids Res. (1987) 15:2891-2909; Connolly, Nucleic Acids Res. (1987) 15:3131-3139; Bischoff et al., Anal. Biochem. (1987) 164:336-344; Blanks et al., Nucleic Acids Res. (1988) 16:10283-10299; U.S. Patent Nos. 4,849,513, 5,015,733, 5,118,800, and 5,118,802). Subsequent to deprotection, the latent amine, thiol, and carboxyl functionalities can be used to covalently attach the PN to a peptide (Benoit et al., Neuromethods (1987) 6:43-72; Sinah et al., Oligonucleotide Analogues: A Practical Approach (1991) IRL Press).

A peptide portion can be attached to a modified cytosine or uracil at any position in the ISS-PN. The incorporation of a "linker arm" possessing a latent reactive functionality, such as an amine or carboxyl group, at C-5 of the modified base provides a handle for the peptide linkage (Ruth, 4th Annual Congress for Recombinant DNA Research, p. 123).

The linkage of the ISS-PN to a peptide can also be formed through a high-affinity, non-covalent interaction such as a biotin-streptavidin complex. A biotinyl group can be attached, for example, to a modified base of an oligonucleotide (Roget et al., Nucleic Acids Res. (1989) 17:7643-7651). Incorporation of a streptavidin moiety into the peptide portion allows formation of a non-covalently bound complex of the streptavidin conjugated peptide and the biotinylated PN.

The linkage of the ISS-PN to a lipid can be formed using standard methods. These methods include, but are not limited to, the synthesis of oligonucleotide-phospholipid conjugates (Yanagawa et al., Nucleic Acids Symp. Ser. (1988) 19:189-92), oligonucleotide-fatty acid conjugates (Grabarek et al., Anal. Biochem. (1990) 185:131-35; Staros et al., Anal. Biochem. (1986) 156:220-22), and oligonucleotide-sterol conjugates (Boujrad et al., Proc. Natl. Acad. Sci. USA (1993) 90:5728-31).

The linkage of the ISS-PN to a oligosaccharide can be formed using standard known

of cells incubated or transfected with an ISS-PN/IMM) as well as systemic or localized routes. However, those of ordinary skill in the art will appreciate that methods and localized routes which direct the ISS-PN/IMM into antigen-sensitized tissue will be preferred in most circumstances to systemic routes of administration, both for immediacy of therapeutic effect and avoidance of *in vivo* degradation.

The entrance point for many exogenous antigens into a host is through the skin or mucosa. Thus, delivery methods and routes which target the skin (e.g., for cutaneous and subcutaneous conditions) or mucosa (e.g., for respiratory, ocular, lingual or genital conditions) will be especially useful. Those of ordinary skill in the clinical arts will be familiar with, or can readily ascertain, means for drug delivery into skin and mucosa. For review, however, exemplary methods and routes of drug delivery useful in the invention are briefly discussed below.

Intranasal administration means are particularly useful in addressing respiratory inflammation, particularly inflammation mediated by antigens transmitted from the nasal passages into the trachea or broncheoli. Such means include inhalation of aerosol suspensions or insufflation of the polynucleotide compositions of the invention. Nebulizer devices suitable for delivery of polynucleotide compositions to the nasal mucosa, trachea and bronchioli are well-known in the art and will therefore not be described in detail here. For general review in regard to intranasal drug delivery, those of ordinary skill in the art may wish to consult Chien, *Novel Drug Delivery Systems*, Ch. 5 (Marcel Dekker, 1992).

Dermal routes of administration, as well as subcutaneous injections, are useful in addressing allergic reactions and inflammation in the skin. Examples of means for delivering drugs to the skin are topical application of a suitable pharmaceutical preparation, transdermal transmission, injection and epidermal administration.

For transdermal transmission, absorption promoters or iontophoresis are suitable

topical cremes and injectable liquids are all examples of suitable mileaus for delivering drugs to the eye.

Systemic administration involves invasive or systemically absorbed topical administration of pharamaceutical preparations. Topical applications as well as intravenous and intramuscular injections are examples of common means for systemic administration of drugs.

2. Dosing parameters

A particular advantage of the ISS-PN/IMM of the invention is their capacity to exert immunomodulatory activity even at relatively minute dosages. Although the dosage used will vary depending on the clinical goals to be achieved, a suitable dosage range is one which provides up to about 1-1000 μg of ISS-PN/IMM/ml of carrier in a single dosage. Alternatively, a target dosage of ISS-PN/IMM can be considered to be about 1-10 μM in a sample of host blood drawn within the first 24-48 hours after administration of ISS-PN/IMM. Based on current studies, ISS-PN/IMM are believed to have little or no toxicity at these dosage levels.

In this respect, it should be noted that the anti-inflammatory and immunotherapeutic activity of ISS-PN/IMM in the invention is essentially dose-dependent. Therefore, to increase ISS-PN/IMM potency by a magnitude of two, each single dose is doubled in concentration. Clinically, it may be advisable to administer the ISS-PN/IMM in a low dosage (e.g., about 1 μg/ml to about 50 μg/ml), then increase the dosage as needed to achieve the desired therapeutic goal.

In view of the teaching provided by this disclosure, those of ordinary skill in the clinical arts will be familiar with, or can readily ascertain, suitable parameters for administration of ISS-PN/IMM according to the invention.

"Prodrugs: Topical and Ocular Drug Delivery" (Marcel Dekker, 1992), and at places elsewhere in the text. All of these references are incorporated herein for the sole purpose of illustrating the level of knowledge and skill in the art concerning drug delivery techniques.

A colloidal dispersion system may be used for targeted delivery of the ISS-PN/IMM to specific tissue. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome.

Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 μm can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, *et al., Trends Biochem. Sci.*, 6:77, 1981). In addition to mammalian cells, liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes encoding the antisense polynucleotides at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, *et al., Biotechniques*, 6:682, 1988).

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be

herein by reference solely to illustrate the standard level of knowledge in the art concerning conjugation of PNs to lipids). Targeted delivery of ISS-PN/IMM can also be achieved by conjugation of the ISS-PN/IMM to a the surface of viral and non-viral recombinant expression vectors, to an antigen or other ligand, to a monoclonal antibody or to any molecule which has the desired binding specificity.

Co-administration of a peptide drug with an ISS-PN/IMM according to the invention may also be achieved by incorporating the ISS-PN/IMM in *cis* or in *trans* into a recombinant expression vector (plasmid, cosmid, virus or retrovirus) which codes for any therapeutically beneficial protein deliverable by a recombinant expression vector. If incorporation of an ISS-PN/IMM into an expression vector for use in practicing the invention is desired, such incorporation may be accomplished using conventional techniques which do not require detailed explanation to one of ordinary skill in the art. For review, however, those of ordinary skill may wish to consult Ausubel, *Current Protocols in Molecular Biology*, *supra*.

D. *Screening for Active ISS-PN/IMM*

Confirmation that a particular compound has the properties of an ISS-PN/IMM useful in the invention can be obtained by evaluating whether the ISS-PN/IMM affects cytokine secretion and IgG antibody isotype production as described in Section A.I, above. Details of *in vitro* techniques useful in making such an evaluation are given in the Examples; those of ordinary skill in the art will also know of, or can readily ascertain, other methods for measuring cytokine secretion and antibody production along the parameters taught herein.

E. *Kits for Use in Practicing the Methods of the Invention*

For use in the methods described above, kits are also provided by the invention. Such kits may include any or all of the following: ISS-PN/IMM (conjugated or

EXAMPLE I

SELECTIVE INDUCTION OF A Th1 RESPONSE IN A HOST AFTER ADMINISTRATION OF AN ISS-PN/IMM

In mice, IgG 2A antibodies are serological markers for a Th1 type immune response, whereas IgG 1 antibodies are indicative of a Th2 type immune response. Th2 responses include the allergy-associated IgE antibody class; soluble protein antigens tend to stimulate relatively strong Th2 responses. In contrast, Th1 responses are induced by antigen binding to macrophages and dendritic cells.

To determine which response, if any, would be produced by mice who received ISS-PN/IMM according to the invention, eight groups of Balb/c mice were immunized with 10μg β-galactosidase protein (conjugated to avidin; Sigma, St. Louis, MO) to produce a model allergic phenotype. As set forth in the Table below, some of the mice received antigen alone, some received an antigen-ISS-PN conjugate or a conjugate using a mutant, non-stimulatory PN as a conjugate for the antigen, and others received the antigen in an unconjugated mixture with an ISS-PN. Naive mice are shown for reference:

shown in FIGURE 2, immunization of the mice with the antigen itself or with the PN/IMM induced production of relatively high titers of IgG 1 antibodies. The data shown in the FIGURES comprise averages of the values obtained from each group of mice.

To evaluate the effect of treatment of a host before and after a secondary antigen challenge, 3 groups of Balb/c mice were immunized with 10μg of antigen E (AgE) in alum to produce a model allergic phenotype and challenged again with the antigen, ISS-PN/IMM or mutant (nonstimulatory) PN/IMM at 5 weeks post-priming. An ELISA for IgG1 and IgG2a antibodies was performed as described 4 weeks after priming (one week before secondary antigen challenge) and again at 7 weeks (2 weeks after secondary challenge).

Again, the mice who received the ISS-PN/IMM mounted a strong Th1 type response to the antigen (IMM) as compared to the antigen-immunized and mutant PN/IMM immunized mice (FIGURE 3), while the reverse was true of a Th2 type response in the same mice (FIGURE 4).

These data indicate that a selective Th1 response is induced by administration of an ISS-PN/IMM according to the invention to both an antigen-primed (pre-antigen challenge) and an antigen-challenged host.

EXAMPLE II

SUPPRESSION OF IgE ANTIBODY RESPONSE TO ANTIGEN BY IMMUNIZATION WITH ISS-PN/IMM

To demonstrate the IgE suppression achieved through stimulation of a Th1 type cellular immune response in preference to a Th2 type cellular immune response, five

(4x105/well) in a medium of RPMI 1640 with 10% fetal calf serum. Supernatants were obtained at days 1, 2 and 3.

Th1 cytokine (INFγ) levels were assayed with an anti-INFγ murine antibody assay (*see*, e.g., Coligan, "*Current Protocols in Immunology*", Unit 6.9.5., Vol. 1, Wiley & Sons, 1994). Relatively low levels of INF-γ would be expected in mice with a Th2 phenotype, while relatively high levels of INF-γ would be expected in mice with a Th1 phenotype.

As shown in FIGURE 5, levels of Th1 stimulated IFN-γ secretion were greatly increased in the ISS-PN/IMM treated mice, but substantially reduced in each other set of mice (as compared to the control), indicating development of a Th2-type phenotype in the latter mice and a Th1 phenotype in the ISS-PN/IMM treated mice.

EXAMPLE IV

BOOSTING OF CTL RESPONSES BY ISS-PN/IMM

A mixture of lymphoytes was obtained and contacted with βgal antigen alone or as part of the constructs and mixtures described in Example I. As shown in FIGURE 6, CTL production in response to ISS-PN/IMM was consistently higher than the response to antigen delivered in other forms; even twice as high than in animals treated with an unconjugated mixture of ISS-PN and IMM antigen.

In the experiment, the higher values for the mice treated with M-ISS-PN/IMM after antigen challenge as compared to the conventionally immunized mice is most likely owing to the antigen carrier properties of DY1019.

Thus, longer-term immunity mediated by cellular immune responses is benefitted by treatment according to the invention.

The invention claimed is:

9. The composition of claim 6, wherein the oligonucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, and AGCGTC.

10. The composition of claim 6, wherein the oligonucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, and GACGTT.

11. The composition of claim 2, wherein the polynucleotide further comprises a linear DNA sequence.

12. The composition of claim 2, wherein the polynucleotide further comprises a circular DNA sequence.

13. The composition of claim 2, wherein the polynucleotide further comprises an RNA nucleotide sequence.

14. The composition of claim 13, wherein the RNA nucleotide sequence comprises a sequence selected from the group consisting of AACGUU, AACGpI, AACGpC, AGCGUC, AGCGpI, AGCGpC, GACGCU, GACGCpI, GACGCpC, GACGUU, GACGpI, GACGpC, GACGUC, GACGpI, GACGpC.

15. The composition of claim 13, wherein the RNA nucleotide sequence comprises a double-stranded poly(I•C) sequence.

16. The composition of claim 13, wherein the RNA nucleotide sequence comprises a sequence selected from the group consisting of AACGUU, AACGpI, AACGpC, AGCGUC, AGCGpI, AGCGpC.

25. The composition of claim 4, wherein the CG containing nucleotide sequence further comprises a modified oligonucleotide.

26. The composition of claim 6, wherein the 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3' nucleotide sequence further comprises a modified oligonucleotide.

27. An immunomodulatory composition comprising an immunomodulatory molecule, which molecule comprises an antigen and an immunostimulatory peptide, conjugated to an polynucleotide that contains at least one ISS.

28. The composition of claim 27, wherein the polynucleotide is DNA or RNA.

29. The composition of claim 27, wherein the immunostimulatory peptide is selected from the group consisting of co-stimulatory molecules, cytokines, chemokines, targeting protein ligands, and trans-activating factors.

30. The composition of claim 27, wherein the ISS comprises a DNA or RNA nucleotide sequence selected from the group CG, p(GC) and p(IC).

31. The composition of claim 27, wherein the ISS comprises a CG containing oligonucleotide.

32. The composition of claim 31, wherein the ISS further comprises a pG nucleotide sequence.

33. The composition of claim 31, wherein the CG containing nucleotide sequence is the nucleotide sequence 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3'.

42. The composition of claim **40**, wherein the RNA nucleotide sequence comprises a double-stranded poly(I•C) nucleotide sequence.

43. The composition of claim **40**, wherein the RNA nucleotide sequence comprises a nucleotide sequence selected from the group consisting of AACGUU, AACGpI, AACGpC, AGCGUC, AGCGpI, AGCGpC.

44. The composition of claim **40**, wherein the RNA nucleotide sequence comprises a nucleotide sequence selected from the group consisting of AACGUU, AACGpI, AACGpC.

45. The composition of claim **29**, wherein the polynucleotide portion further comprises at least one modified oligonucleotide.

46. The composition of claim **38**, wherein the ISS is contained within the linear DNA nucleotide sequence, and further wherein the ISS comprises a Purine, Purine, CG, Pyrimidine, Pyrimidine nucleotide sequence.

47. The composition of claim **38**, wherein the ISS is contained within the linear DNA nucleotide sequence, and further wherein the ISS comprises a CG containing pG nucleotide sequence.

48. The composition of claim **39**, wherein the ISS is contained within the circular DNA nucleotide sequence, and further wherein the ISS comprises a Purine, Purine, CG, Pyrimidine, Pyrimidine nucleotide sequence.

49. The composition of claim **39**, wherein the ISS is contained within the circular DNA nucleotide sequence, and further wherein the ISS comprises a CG containing pG nucleotide sequence.

58. The method of claim **57**, wherein the ISS comprises a DNA or RNA nucleotide sequence selected from the group CG, p(GC) and p(IC).

59. The method of claim **57**, wherein the ISS comprises a CG containing oligonucleotide.

60. The method of claim **59**, wherein the ISS further comprises a pG nucleotide sequence.

61. The method of claim **59**, wherein the CG containing nucleotide sequence is the nucleotide sequence 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3'.

62. The method of claim **59**, wherein the CG containing nucleotide sequence is a palindromic or non-palindromic oligonucleotide nucleotide sequence.

63. The method of claim **59**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, AGCGTC, GACGTC, GGCGTC, AACGCC, AGCGCC, GACGCC, GGCGCC, AACGCT, AGCGCT, GACGCT, and GGCGCT.

64. The method of claim **59**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, and AGCGTC.

65. The method of claim **59**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, and GACGTT.

66. The method of claim **54**, wherein the immune response modulation comprises the induction of a Th1 response.

76. A method of modulating an immune response comprising the administration of an immunomodulatory composition comprising an immunomodulatory molecule, which molecule is comprised of an antigen and an immunostimulatory peptide, conjugated to an polynucleotide that contains at least one ISS.

77. The method of claim 76, wherein the route of administration is a dermal route.

78. The method of claim 76, wherein the route of administration is low-frequency ultrasonic delivery.

79. The method of claim 76, wherein the immunostimulatory peptide is selected from the group consisting of co-stimulatory molecules, cytokines, chemokines, targeting protein ligands, and trans-activating factors.

80. The method of claim 79, wherein the ISS comprises a nucleotide sequence selected from the group CG, p(GC) and p(IC).

81. The method of claim 79, wherein the ISS comprises a CG containing oligonucleotide.

82. The method of claim 81, wherein the ISS further comprises a pG nucleotide sequence.

83. The method of claim 81, wherein the CG containing nucleotide sequence is the nucleotide sequence 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3'.

93. The method of claim **91**, wherein the CG containing nucleotide sequence is the nucleotide sequence 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3'.

94. The method of claim **91**, wherein the CG containing nucleotide sequence is a double-stranded palindromic or single-stranded non-palindromic oligonucleotide nucleotide sequence.

95. The method of claim **91**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, AGCGTC, GACGTC, GGCGTC, AACGCC, AGCGCC, GACGCC, GGCGCC, AACGCT, AGCGCT, GACGCT, and GGCGCT.

96. The method of claim **91**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, and AGCGTC.

97. The method of claim **91**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, and GACGTT.

98. A method for introducing a soluble antigen into the Class I MHC processing pathway of the mammalian immune system to elicit a CTL response to the antigen comprising administering a polynucleotide conjugated to an immunomodulatory molecule, which molecule comprises the antigen, to a mammalian host.

99. The method of claim **98** wherein the polynucleotide includes at least one ISS.

100. The method of claim **98** wherein the polynucleotide is free of ISS.

110. The method of claim **98** wherein the polynucleotide comprises a GpG oligonucleotide.

111. The method of claim **110**, wherein the nucleotide sequence is selected from the group consisting of AAGGTT, AGGGTT, GAGGTT, GGGGTT, AAGGTC, AGGGTC, GAGGTC, GGGGTC, AAGGCC, AGGGCC, GAGGCC, GGGGCC, AAGGCT, AGGGCT, GAGGCT, and GGGGCT.

112. The composition of claim **110**, wherein the nucleotide sequence is selected from the group consisting of AAGGTT, AGGGTT, GAGGTT, GGGGTT, AAGGTC, and AGGGTC.

113. The composition of claim **110**, wherein the nucleotide sequence is selected from the group consisting of AAGGTT, AGGGTT, and GAGGTT.

114. A composition for introducing a soluble antigen into the Class I MHC processing pathway of the mammalian immune system to elicit a CTL response to the antigen comprising a polynucleotide conjugated to an immunomodulatory molecule, which molecule comprises the antigen.

115. The composition of claim **114**, wherein the antigen is selected from the group consisting of proteins, glycoproteins and polysaccharides.

116. The composition of claim **114** wherein the polynucleotide comprises a GpG oligonucleotide.

117. The composition of claim **116**, wherein the nucleotide sequence is selected from the group consisting of AAGGTT, AGGGTT, GAGGTT, GGGGTT, AAGGTC, AGGGTC, GAGGTC, GGGGTC, AAGGCC, AGGGCC, GAGGCC, GGGGCC, AAGGCT, AGGGCT, GAGGCT, and GGGGCT.

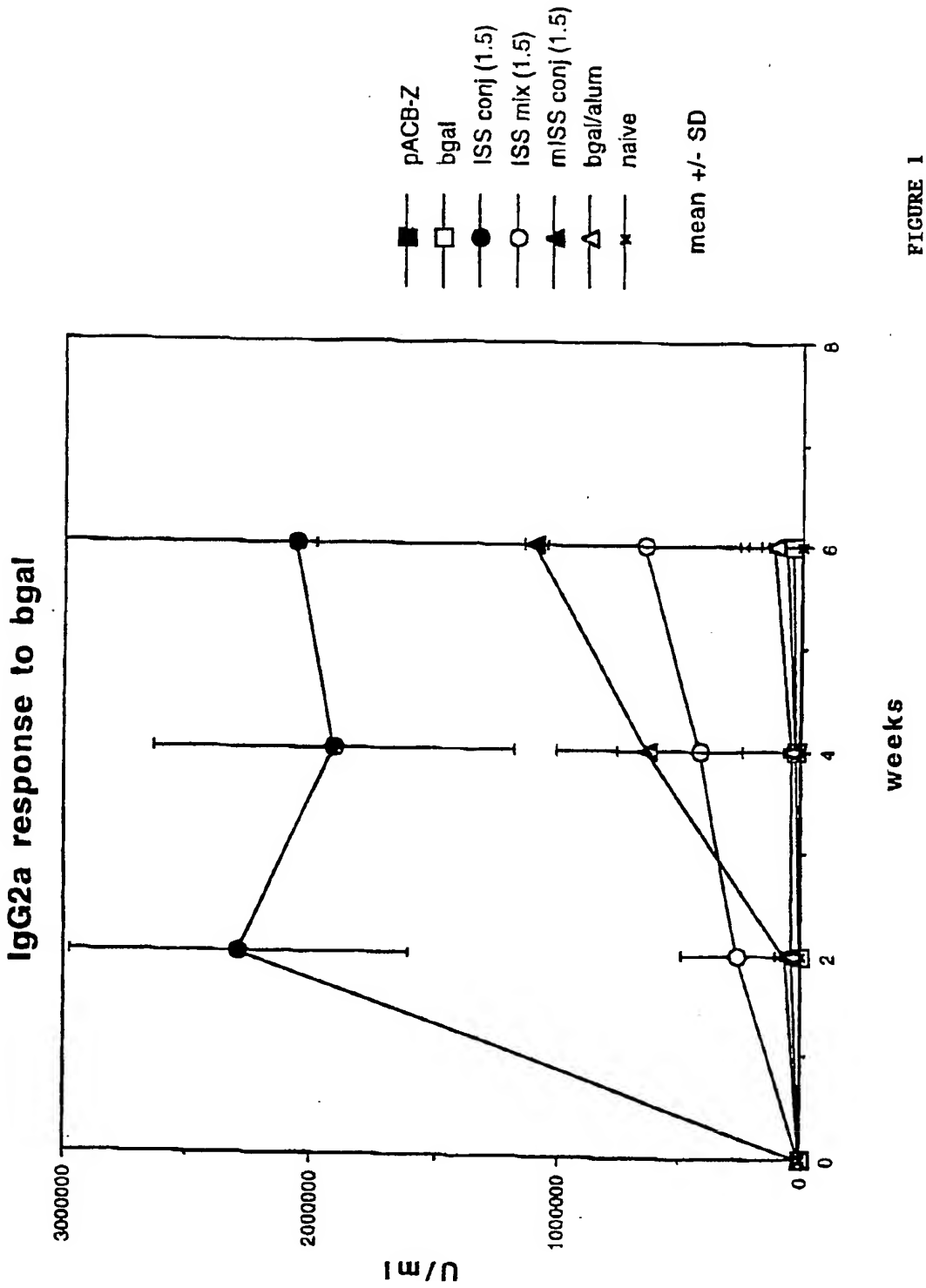
IgG2a response to bgal
U/ml
3000000
2000000
1000000
0
0
2
4
6
8
weeks
pACB-Z
bgal
ISS conj (1.5)
ISS mix (1.5)
mISS conj (1.5)
bgal/alum
naive
mean +/- SD

FIGURE 1

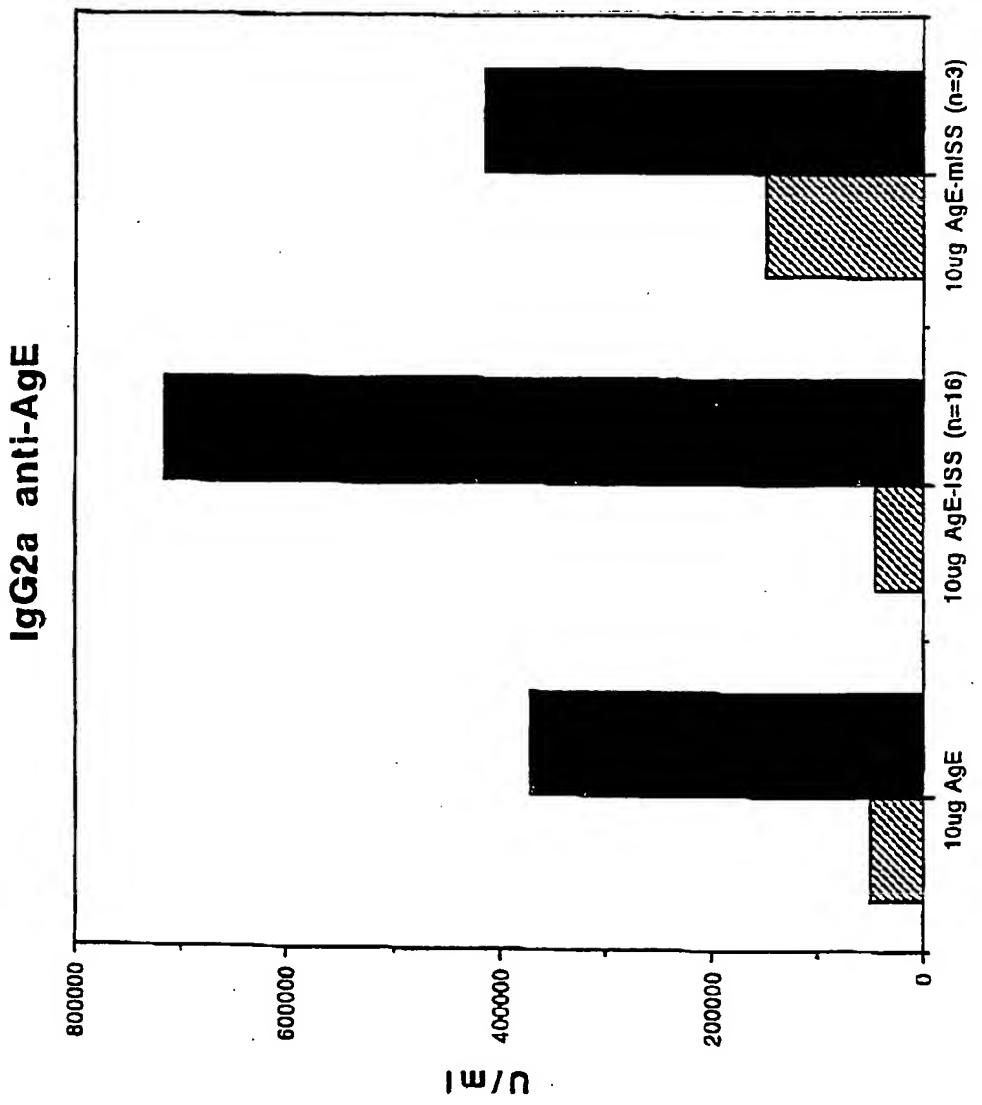
IgG2a anti-AgE
U/ml
800000
600000
400000
200000
0
wk 4
wk 7
10ug AgE
10ug AgE-ISS (n=16)
10ug AgE-mISS (n=3)

FIGURE 3

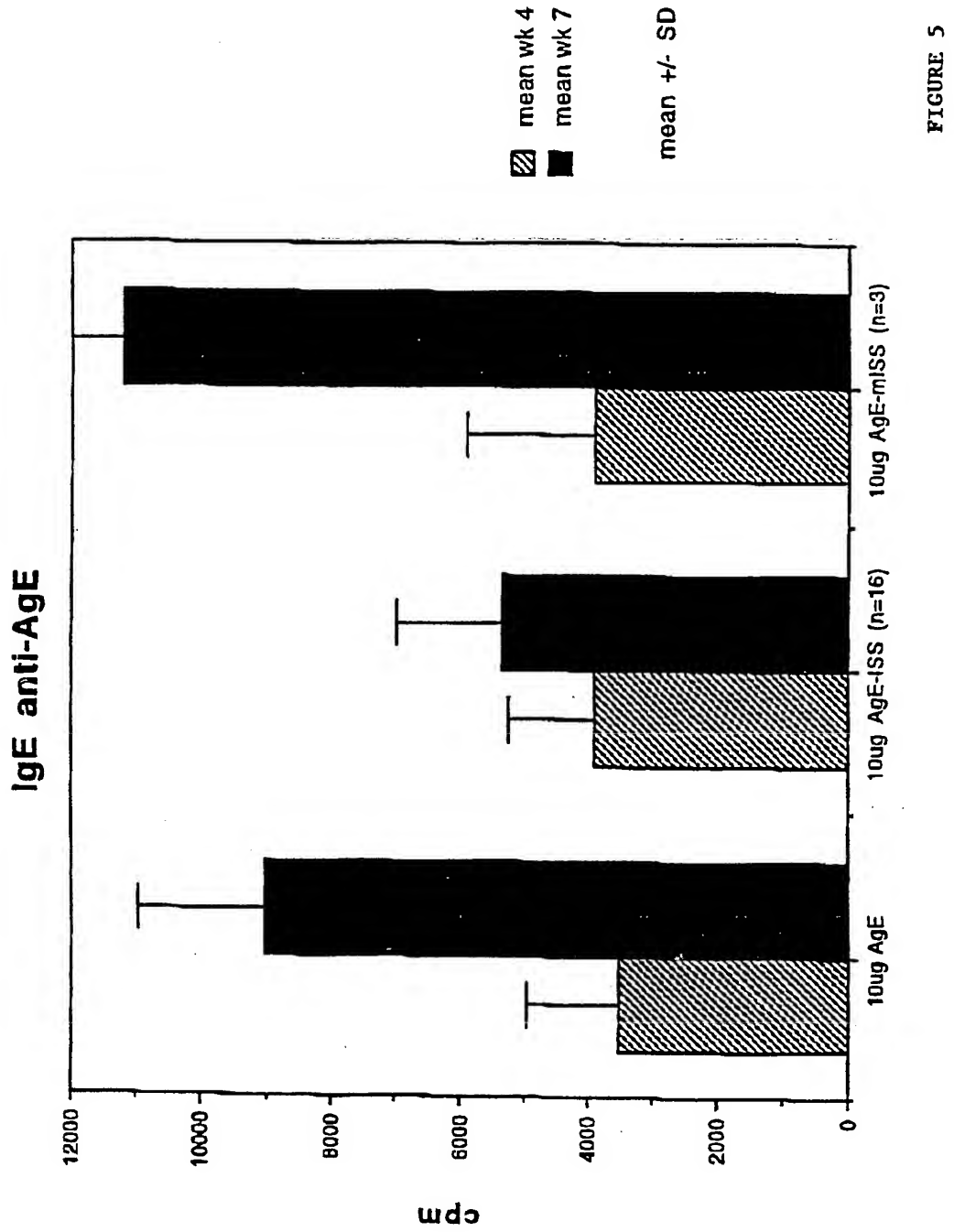
IgE anti-AgE
cpm
12000
10000
8000
6000
4000
2000
0
mean wk 4
mean wk 7
mean +/- SD
10ug AgE
10ug AgE-lSS (n=16)
10ug AgE-mISS (n=3)

FIGURE 5

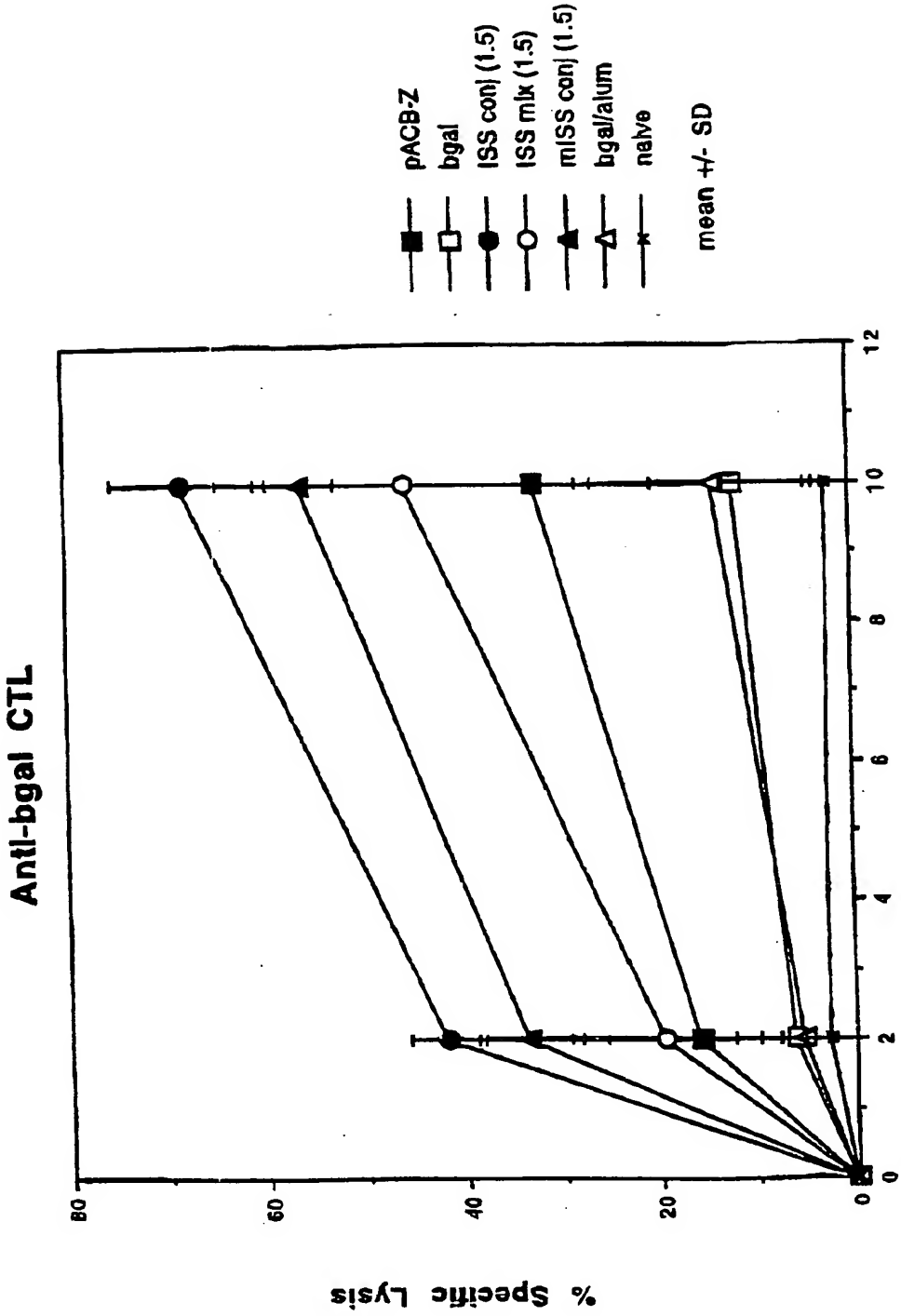
Anti-bgal CTL
% Specific Lysis
E:T ratio
pACB-Z
bgal
ISS conj (1.5)
ISS mix (1.5)
mISS conj (1.5)
bgal/alum
naive
mean +/- SD
0
20
40
60
80
0
2
4
6
8
10
12

FIGURE 7

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RAZ E. ET AL: "Potential role of immunostimulatory DNA sequences (ISS) in genetic immunization and autoimmunity" ARTHRITIS & RHEUMATISM, vol. 39, no. 9, September 1996, page 615 XP002058356 see the whole document ---	1-119
A	SATO Y. ET AL: "Immunostimulatory DNA sequences necessary for effective intradermal gene immunization" SCIENCE, vol. 273, July 1996, LANCASTER, PA US, XP002058357 see the whole document ---	1-119
A	ARTHUR M. KRIEG ET AL: "CpG motifs in bacterial DNA trigger direct B-cell activation" NATURE, vol. 374, 1995, LONDON GB, pages 546-549, XP002058358 see the whole document ---	1-119
A	BALLAS, Z.K. ET AL: "Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA" JOURNAL OF IMMUNOLOGY, vol. 157, September 1996, BALTIMORE US, pages 1840-1845, XP002058359 see the whole document ---	1-119
A	RAZ E. ET AL: "Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 93, May 1996, WASHINGTON US, pages 5141-5145, XP002058360 see the whole document ---	1-119
A	BRANDA R.F. ET AL: "Amplification of antibody production by phosphorothioate oligodeoxynucleotides" THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE, vol. 128, no. 3, September 1996, pages 329-338, XP002058361 see the whole document ---	1-119
A	WO 95 26204 A (ISIS PHARMACEUTICALS INC) 5 October 1995 see the whole document ---	1-119

-/--

INTERNATIONAL SEARCH REPORT

Int...ational application No.
PCT/US 97/19004

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern ial Application No
PCT/US 97/19004

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